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Full Length Research Paper

Genetic diversity and identification of variety-specific AFLP markers in fenugreek (*Trigonella foenum*-graecum)

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Fenugreek (*Trigonella foenum-graecum*), an important spice crop belongs to the family Fabaceae. India is the largest producers as well as the largest exporter of fenugreek. Five common fenugreek varieties of India were included in this study for molecular analysis. For randomly amplified polymorphic DNA (RAPD) analysis, a total of 47 bands in the size range of 200 to 5000 bp were produced by examining across genotypes with 9 RAPD primers, with an average polymorphism of 62.4%. Total numbers of bands ranged from 3 (U407) to 7 (OPB-15). With 17 fluorescently labeled amplified fragment length polymorphism (AFLP) primer combinations (PCs), a total of 669 peaks in the size range of 50 to 538 bp were amplified. Total numbers of bands ranged from 21 (E-AAC/M-CT) to 60 (E-AAC/M-CAC). The mean genetic diversity (Nei's 1973) across all loci was found to be 23.83 and 2.1% with RAPD and AFLP markers, respectively. In all the fenugreek varieties, a total of 25 variety-specific AFLP markers were found. Phylogenetic trees among 5 plant varieties were constructed based on Nei's coefficient standard genetic distances using unweighted pair group method with arithmetic mean (UPGMA) method. For RAPD and AFLP analysis, Gujarat Methi-1 (GM-1) and Gujarat Methi-2 (GM-2) clustered together showed more similarities than other varieties. In fenugreek, RAPD markers were found more polymorphic than AFLP markers. RAPD markers also showed more diversity in comparison to AFLP markers, although the data generated for AFLP markers were more authenticated and reproducible than RAPD markers.

Key words: AFLP, RAPD, genetic diversity, automated genetic analyzer, polymorphism.

INTRODUCTION

Fenugreek (*Trigonella foenum-graecum*) popularly known as “Methi” belongs to family Fabaceae and subfamily Papilionaceae. It is an important spice crop largely grown in the northern India during rabi season. Among the spices, it is one of the oldest plants, originating in India and Northern Africa and widely used as a condiment, a dye and in medicines (Basch et al., 2003). India is the largest producer of fenugreek in the world, with Rajasthan, Gujarat, Uttarakhand, Uttar Pradesh, Madhya Pradesh, Maharashtra, Haryana and Punjab being the major fenugreek producing states. On the basis of height character, two fairly distinct types of fenugreek plants are recognized, the dwarf type grown for culinary purposes and the tall type grown for medicinal purposes (Zargar et al., 1992). Women (mothers) have used the spice fenugreek as a galactagogue (an agent that encourages or increases the secretion of milk) since ancient times in the Middle East, North Africa, and India, and it has been observed to increase breast milk production within 24 to 72 h after taking the herb (www.babycenter.com). Fenugreek is commonly used in the Ayurveda and Unani medicines (Srinivasan, 2006).

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Abbreviations: **RAPD**, Randomly amplified polymorphic DNA; **AFLP**, amplified fragment length polymorphism; **UPGMA**, unweighted pair group method with arithmetic mean; **GM**, Gujarat Methi.

This herb is mainly used for the treatment of diabetes and blood sugar level (Zargar et al., 1992; Mohamad et al., 2004), cancer (Shabbeer et al., 2009), poor digestion and gastric inflammation (Ismail, 2009), women problems like as menstrual, labor pains as well as uterus and breast development (www.globalherbalsupplies.com).

In India, fenugreek is grown as a winter season crop for commercial seed production. Generally, two types of fenugreek (methi) are grown in India: Common methi and kasuri methi. The Common methi is a fast growing herb producing upright shoots to a height of 40 to 70 cm. Two to three white flowers are produced in axils of each leaf. On the other hand, Kasuri methi is a slow growing herb with a characteristic fragrance. It produces bright orange to yellow flowers. All five varieties used in the present study are improved varieties of common methi in terms of high yield, high quality, dual purpose types (suitable for seed purpose as well as green purpose), early maturing types, bold grains and short plant types. Rmt-1 (Rajasthan Methi-1) is a pure line selection released from Jobner, Rajasthan in 1989. Duration of the crop is 145 days and yield per ha is 1500 kg grain. Gujarat Methi-1 (GM-1) and Gujarat Methi-2 (GM-2) varieties were released by Centre for Research on Seed Spices, S. D. Agricultural University, Jagudan, Gujarat. GM-1 was developed in the year 1999 with the aim of increasing the yield from 1034 to 1138 kg/ha, while GM-2 is an improved high yielding disease resistant variety. Pusa Early Bunching (PEB) is an early variety developed at IARI, New Delhi. It takes about 125 days to maturity. MMT-5 is a local variety of Maharastra state.

Molecular markers have opened exciting new windows through which to view the natural biological world. Among the molecular markers, DNA markers have been successfully applied for genome identification (Rafalski and Tingey, 1993; Plomion et al., 1995), molecular characterization (Singh et al., 2010; Ijaz and Khan, 2009), phylogenetic relationships studies among various plant varieties (Martos et al., 2005) and in development of unique molecular signatures (Sudheer-Pamidimarri et al., 2009). DNA based markers are generally classified as hybridization-based markers and polymerase chain reaction (PCR)-based markers. In hybridization based markers, DNA profiles are visualized by hybridizing the restriction enzyme-digested DNA to a labeled probe, which is a DNA fragment of known origin or sequence for example the restriction fragment length polymorphism (RFLP). While polymerase chain reaction (PCR)-based markers involve *in vitro* amplification of particular DNA sequences or loci with the help of specifically or arbitrarily chosen oligonucleotide sequences (primers) and a thermostable DNA polymerase enzyme. The amplified fragments are separated electrophoretically and analyzed by computer softwares. Due to the non-availability of sequence based markers in fenugreek, arbitrary primed PCR techniques that are developed without prior sequence information such as random amplification of polymorphic DNA (RAPD), inter simple sequence repeat(ISSR) and amplified fragment length polymerphism (AFLP) may play a big role in molecular characterization, as well as in the identification and authentication of fenugreek varieties.

MATERIALS AND METHODS

Seeds of five plant varieties of fenugreek were collected from Rajasthan, Gujarat, Uttarakhand and Maharashtra states of India. These varieties were Rajasthan Methi-1 (Rmt-1), Gujarat Methi-1 (GM-1), Gujarat Methi-2 (GM-2), Pusa Early Bunching (PEB) and MMT-5. Seeds were collected from single plant for each variety to avoid cross contamination.

DNA isolation

Seed samples soaked in Petri plate for a week were used for DNA extraction. DNA was isolated from the seeds using modified cetyltrimethylammonium bromide (CTAB) method described by Saghai-Maroof et al. (1984), and its quality and quantity were analyzed using 1% agarose gel electrophoresis and an ND-8000 spectrophotometer (NanoDropTechnologies, USA).

RAPD assay

RAPD assay performed with random decamer primers obtained from DNA amplification was done using 9 RAPD primers (Table 1). Polymerase chain reaction (PCR) was carried out in a volume of 25 µL containing 1X Reaction buffer with 2.0 mM $MgCl_2$, 10 pM primer, 200 µM each of deoxynucleotides (dNTPs), 1 unit of *Taq* polymerase and 50 ng of genomic DNA. The PCR mixture was subjected to initial denaturation of 94°C for 5 min. The reaction was subjected to the 40 cycles of initial denaturation of 94°C for 1 min, 36°C for 1 min and 72°C for 2 min and annealing with a final extension of 72°C for 7 min. The products of amplification were stored at 4°C till further usage.

Amplified products along with external size standard were separated in a horizontal gel electrophoresis unit using 1.5 % agarose gel in 1 X TAE buffer and stained with ethidium bromide. The banding patterns was visualized under UV light and photographed using a Gel Documentation System. The number and intensity of monomorphic and polymorphic bands were recorded. The PCR was repeated at least twice in order to check reproducibility. The amplification profiles of those primers that produced amplification with all samples only, were used in the final analysis.

AFLP assay

The AFLP assay was performed as previously described by Vos et al. (1995) and Vuylsteke et al. (2007) with minor modifications. To produce *Eco*RI/*Mse*I AFLP markers, genomic DNA (500 ng) was digested using the restriction endonuclease enzymes *Eco*RI and *Mse*I (5 U each), and double-stranded *Eco*RI and *Mse*I adapters were ligated to the ends of DNA fragments. This was served as template for the pre-amplification reaction. Pre-amplification was carried out using *Eco*RI primer with one selective nucleotide (5´-GACTGCGTACCAATTC**A**-3´) and *Mse*I primer with one selective nucleotide (5´-GATGAGTCCTGAGTAA**C**-3´). For pre-amplification reaction, 5 µL of adapter ligated DNA was then added to a pre-amplification mix containing all PCR components with *Eco*RI and *Mse*I pre-amplification primers (carrying one selective nucleotide each). The PCR reaction was performed for 25 cycles with the

Table 1. Genetic diversity parameters for RAPD markers.

S/N	Primer	Sequence (5'-3')	Polymorphic band	Total number of band	Polymorphism (%)
1	OPA03	AGT CAG CCA C	3	5	60.0
2	OPB07	GGT GAC GCA G	6	8	75.0
3	OPC19	GTT GCC AGC C	6	6	100.0
4	OPF09	CCA AGC TTC C	3	4	75.0
5	OA04	AAT CGG GCT G	5	5	100.0
6	U388	CGG TCG CGT C	2	5	40.0
7	U407	TGG TCC TGG C	1	3	33.3
8	OPB15	GGA GGG TGT T	2	7	28.6
9	OB10	CTG CTG GGA C	2	4	50.0
Total			30	47	561.9
Average			3.3	5.2	62.4

following cycle profile: denaturation at 94°C for 30 s, annealing at 56°C for 60 s, extension at 72°C for 60 s, with the final extension at 72°C for 5 min. The pre-amplification reaction product was diluted 20-fold with T:E (10:1) buffer for further use. This was served as templates for the final selective amplification reactions using primers with two/three selective bases in one or both primers. The selective *Eco*RI primers were fluorescently labeled with 6-FAM. Each selective amplification PCR reaction had total volume of 20 µL containing 5 µL of diluted template DNA, 1X Reaction buffer (50 mM KCl, 10 mM Tris-HCl, pH-8.8, 0.1% Triton X-100 and 0.01% gelatin), 1.5 mM $MgCl_2$, 200 µM of each dNTP (dATP, dGTP, dCTP and dTTP), 1 U of *Taq* polymerase and 5 pmol appropriate selective *Eco*RI primer along with 10 pmol appropriate selective *Mse*I primer. Selective amplification was carried out by 2 cycles at 94°C for 30 s, 66°C for 30 s and 72°C for 60 s followed by a touch-down cycling protocol where the annealing temperature was reduced by 2°C (64, 62, 60, 58 and 56°C) after every two cycles. This continued till the annealing reached 56°C, and finally 25 cycles at 94°C for 30 s, 56°C for 30 s and 72°C for 60 s. The last cycle had an extension time of 5 min. After PCR, the final product was stored at -20°C till further process. For genotyping, all PCR generated products were run on ABI 3730xl genetic analyzer using 0.5 µL of PCR product by mixing with 10.2 µL of HiDi formamide and 0.3 µL of Liz size standard. This mixture was denatured on 95°C before loading the 96 well plate on automated genetic analyzer.

Data analysis

The RAPD bands were scored as 1 for present or 0 for absent across the genotypes and only those that were well defined and consistently repeatable in two independent amplifications were included in the final analysis. All clear and intense bands were scored for the construction of the data matrix. For AFLP, genotyping data was collected in the form of peaks using Data Collection software of ABI (Applied Biosystems) and analysis of AFLP peaks for sizing and scoring were performed using GeneMapper Software Version 4.0 of ABI (Applied Biosystems). The amplification products were scored for the presence (1) and absence (0) of peaks across the genotypes. The data were scored in an Excel sheet and was converted manually in a text format for PopGene software. Fragment analysis was carried out for peaks in the range of 50 to 550 bp.

POPGENE version 1.32 (Yeh et al., 1999) was used to calculate all genetic parameters. Variety-specific RAPD and AFLP markers were scored on the basis of gene frequency. The genetic relationship between the populations was determined by calculating genetic distances (GD) and genetic identities (GI) for all possible population pairs as per Nei's coefficient (1972). Cluster analysis was performed using the unweighted pair group method with arithmetic averages (UPGMA). Dendrograms were constructed using the UPGMA algorithms in the MEGA 4.0 software (Tamura et al., 2007).

RESULTS

Genetic diversity analysis

In the RAPD analysis, 30 primers were used for polymorphism screening, out of which only 9 primers were found polymorphic. A total of 47 bands in the size range of 200 to 5000 bp were produced by examining across genotypes with 9 RAPD primers, with an average polymorphism of 62.4% (Table 1). Total number of bands ranged from 3 (U407) to 7 (OPB-15). The mean Nei's genetic diversity (1973) across all loci was found to be 23.83 % with standard deviation of 0.1913.

In the AFLP analysis, we used 30 primer combinations (PCs) for genotyping. Out of the 30 PCs, 17 PCs were found polymorphic. A total of 669 peaks in the size range of 50 to 538 bp were amplified for 17 PCs with 5 samples (Table 2). Total number of bands ranged from 21 (E-AAC/M-CT) to 60 (E-AAC/M-CAC). The levels of polymorphism were calculated based on the percentage of polymorphic bands, which varied from 1.7% for the E-AAC/M-CAC primer combination to 20.0% for the E-AGC/M-CTG primer combination. All monomorphic and polymorphic peaks were included in the analysis. Out of 669 peaks, 38 were found polymorphic with 6.4% of total polymorphism among 5 samples. The mean Nei's genetic diversity (1973) across all loci was found quite low (2.1%), with standard deviation 0.0876. This indicates that all 5 varieties had a very low polymorphism or less diverse from each other.

Table 2. Genetic diversity parameters for AFLP markers.

S/N	Primer combination	Size range (bp)	Polymorphic peak	Number of peak	Polymorphism rate (%)
1	E-AAC/M-CAA	62 - 471	2	36	5.6
2	E-AAC/M-CAC	50 - 503	1	60	1.7
3	E-AAC/M-CAG	58 - 538	3	37	8.1
4	E-AAC/M-CTC	52 - 377	1	25	4.0
5	E-AAC/M-CTT	57 - 317	2	44	4.5
6	E-AAG/M-CAA	51 - 430	4	35	11.4
7	E-AAG/M-CAC	55 - 377	2	47	4.3
8	E-AAG/M-CAG	59 - 349	1	43	2.3
9	E-AAG/M-CAT	54 - 410	1	47	2.1
10	E-AAG/M-CTA	51 - 301	1	35	2.9
11	E-AGC/M-CAG	74 - 537	2	35	5.7
12	E-AGC/M-CAT	54 - 386	1	34	2.9
13	E-AGC/M-CTG	75 - 531	6	30	20.0
14	E-AGC/M-CTT	51 - 338	1	43	2.3
15	E-AAC/M-CT	57 - 377	4	21	19.0
16	E-AGC/M-CA	50 - 538	1	46	2.2
17	E-AGC/M-CT	56 - 338	5	51	9.8
Total			38	669	108.8
Average			2.23	39.35	6.4

Table 3. Variety-specific AFLP markers in 5 fenugreek varieties with sizes in base pairs (bps).

S/N	Primer combination	Genotype				
		GM-1	GM-2	PEB	MMT-5	Rmt-1
1	E-AAC/M-CAA	268	263		-	-
2	E-AAC/M-CAG	138	60	-	151	-
3	E-AAC/M-CTC	-	-	-	-	275
4	E-AAC/M-CTT	-	-	-	-	232
5	E-AAG/M-CAA	-	-	-	-	275 and 279
6	E-AAG/M-CAC	-	268	267	-	-
7	E-AAG/M-CAT	266	-	-	-	-
8	E-AGC/M-CAG	-	-	-	360	362
9	E-AGC/M-CTG	-	-	180	88, 220 and 241	-
10	E-AAC/M-CT	-	-	179	267	268
11	E-AGC/M-CA	-	-	175	-	-
12	E-AGC/M-CT	273 and 277	-	-	220	-
Total		5	3	4	7	6

Species-specific markers

The variety-specific RAPD marker was not shown due to its low reproducibility. Variety-specific AFLP markers were identified for all 5 plant varieties (5 for GM-1, 3 for GM-2, 4 for PEB, 7 for MMT-5 and 6 for Rmt-1). A total of 25 variety-specific AFLP markers were found with 12 polymorphic primer combinations. The specific markers with sizes in base pairs are shown in Table 3.

Similarity analysis

The genetic similarities and genetic distances were calculated for all possible population pairs using RAPD data (Table 4) and AFLP data (Table 5) as per Nei's coefficient (1972). In RAPD analysis, all population pairs showed a moderate range of similarities about 51 to 89%. Population pair of GM-1 and GM-2 showed minimum genetic distance (GD = 0.1125) and population pair MMT-

Table 4. Nei's original measures of genetic similarity (above diagonal) and genetic distance (below diagonal) as per Nei (1972) for RAPD analysis.

Variety	GM-1	GM-2	PEB	MMT-5	Rmt-1
GM-1	*****	0.8936	0.7021	0.5532	0.7447
GM-2	0.1125	*****	0.7660	0.6170	0.8085
PEB	0.3536	0.2666	*****	0.7234	0.7021
MMT-5	0.5921	0.4829	0.3238	*****	0.5106
Rmt-1	0.2948	0.2126	0.3536	0.6721	*****

Table 5. Nei's Original Measures of genetic similarity (above diagonal) and genetic distance (below diagonal) as per Nei 1972 for AFLP analysis.

Variety	GM-1	GM-2	PEB	MMT-5	Rmt-1
GM-1	*****	0.9791	0.9746	0.9731	0.9716
GM-2	0.0211	*****	0.9746	0.9731	0.9776
PEB	0.0257	0.0257	*****	0.9716	0.9731
MMT-5	0.0273	0.0273	0.0288	*****	0.9686
Rmt-1	0.0288	0.0227	0.0273	0.0319	*****

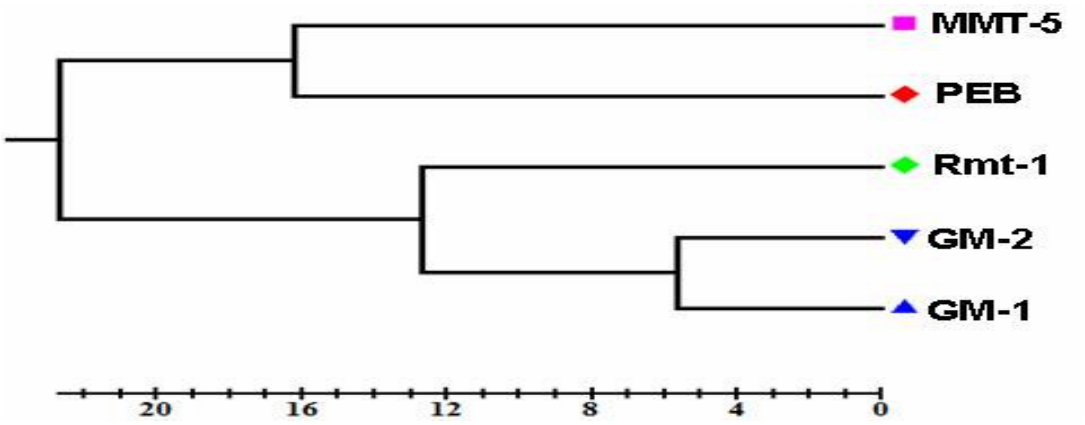

Figure 1. Phylogenetic tree based on Nei's (1972) standard genetic distances using UPGMA method generated for RAPD data.

5 and Rmt-1 showed maximum genetic distance (GD = 0.6721). In AFLP analysis, all population pairs showed a high range of similarities about 97 to 98%. Population pair GM-1 and GM-2 showed minimum genetic distance (GD = 0.0211) and population pair MMT-5 and Rmt-1 showed maximum genetic distance (GD = 0.0319).

Clustering analysis

Phylogenetic trees among 5 plant varieties were constructed based on Nei's coefficient (1972) standard genetic distances using UPGMA (Unweighted Pair Group Method with Arithmetic Mean) method. For RAPD analysis at 77% genetic similarity, the cluster diagram indicated 2 major groups (Figure 1). In the first group, two varieties of fenugreek MMT-5 and PEB were clustered together. The second group consisted of Rmt-1, GM-2 and GM-1. At 87% genetic similarity, this group was divided again into two subgroups, one sub group with Rmt-1 and other with GM-1 and GM-2.

For AFLP analysis, the cluster diagram indicated 4 major groups (Figure 2). In the first group, two varieties of fenugreek (GM-1 and GM-2) clustered together, showing 99% similarity. The second group consisted of PEB, third group consisted of Rmt-1 and fourth group consisted of MMT-5. All 4 groups were tightly closed together. All 5 varieties showed about 97% genetic similarity and were found closely related to each other at genetic level. The results clearly showed that GM-1 and GM-2 clustered together and are more similar than other varieties. RAPD markers showed more diversity as compared with AFLP

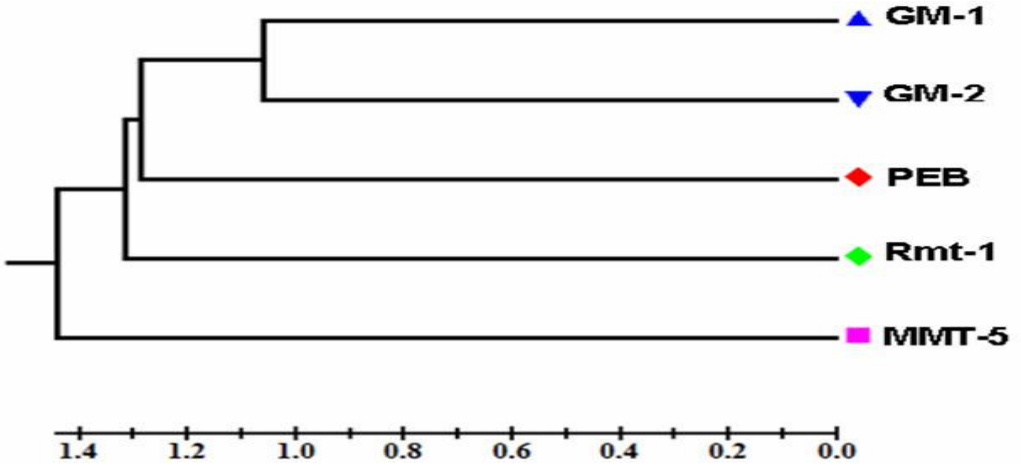

Figure 2. Phylogenetic tree based on Nei's (1972) standard genetic distances using UPGMA method generated for AFLP data.

markers.

DISCUSSION

Fenugreek *(T. foenum-graecum* L.) is an annual and self-pollinated crop. Self-pollination tends to decrease the genetic diversity (low heterozygosity) in a population. Populations with more genetic diversity are able to adapt to change more rapidly than those with less genetic diversity. DNA-based markers provide precise information on genetic diversity and identification of variety-specific markers because of the independence of the confounding effects of environmental factors (Powell et al., 1995). In the non-availability of sequence based markers, random primers such as RAPD, ISSR and AFLP markers are the marker of choice. RAPD markers are arbitrary decamers, which randomly screen various regions of the genomic DNA.

In this study, RAPD marker system revealed high levels of polymorphism of about 62.4% among the fenugreek varieties, which was found vary close to 64.70% polymorphism detected in accessions from India by Kakani et al. (2011) and lower than 70.12% polymorphism detected in accessions of fenugreek from different countries by Dangi et al. (2004). Often, RAPD markers have been considered as undependable due to their low reproducibility for being random and because they are based on small decamer primers (Shasany et al., 2005). In AFLP analysis, a low level of polymorphism of about 6.4% was detected among all fenugreek varieties. Although, the level of polymorphism offered by AFLPs was lower than RAPDs, AFLP markers were much more reliable and provided a more detailed coverage throughout the genome than RAPD because of automated capillary gel electrophoresis and restriction digestion/adaptor ligation techniques used in AFLP analyzing that allows a more precise identification and accurate sizing of similar and dissimilar alleles (Liu et al., 2003).

Overall, a total of 25 variety-specific AFLP alleles were found for all 5 fenugreek Indian varieties. This number is much higher than the eight specific RAPD alleles detected in exotic accessions of fenugreek by Dangi et al. (2004). RAPD has been widely used for diversity analysis in fenugreek by Sundaram and Purwar (2011), Kakani et al. (2011) and Dangi et al. (2004). In fenugreek, no publication was found for genetic diversity analysis using AFLP marker system. Although, AFLP markers were used in some other important spice crops such as black cumin (*Bunium persicum*) by Pezhmanmehr et al. (2009), black pepper by Joy et al. (2007) and Iranian cumin (*Cuminum cyminum*) by Kermani et al. (2006). In UPGMA analysis using both RAPD and AFLP marker system, GM-1 and GM-2 varieties were observed genetically more similar and grouped together. In addition, RAPDs and AFLPs markers confirm their relationships efficiently as both were collected from same geographical region.

In general, both RAPD and AFLP techniques may provide useful information on the level of polymorphism and diversity in fenugreek. In RAPD analysis, however, the reliability and reproducibility of data is a common problem, although, it is relatively cheap and an easy technique than AFLP. Moreover, AFLP marker analysis used to determine the genetic diversity and variety-specific markers in plants is a reliable procedure when coupled with a separation system capable of resolving DNA fragments of 50 to 550 bp with one-base-pair resolution.

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